(37) Transplant rejection.

Prolonged Allograft Survival in TNF Receptor 1-Deficient Recipients Is Due to Immunoregulatory Effects, Not to Inhibition of Direct Antigraft Cytotoxicity[1]

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TNF-α and lymphotoxin (LT)α have been shown to be important mediators of allograft rejection. TNF-R1 is the principal receptor for both molecules. Mice with targeted genetic deletions of TNF-R1 demonstrate normal development of T and B lymphocytes but exhibit functional defects in immune responses. However, the role of TNF-R1-mediated signaling in solid organ transplant rejection has not been defined. To investigate this question, we performed vascularized heterotopic allogeneic cardiac transplants in TNF-R1-deficient (TNF-R1$^{-/-}$) and wild-type mice. Because all allografts in our protocol expressed TNF-R1, direct antigraft effects of TNF-α and LTα were not prevented. However, immunoregulatory effects on recipient inflammatory cells by TNF-R1 engagement was eliminated in TNF-R1$^{-/-}$ recipients. In our study, cardiac allograft survival was significantly prolonged in TNF-R1$^{-/-}$ recipients. Despite this prolonged allograft survival, we detected increased levels of CD8 T cell markers in allografts from TNF-R1$^{-/-}$ recipients, suggesting that effector functions, but not T cell recruitment, were blocked. We also demonstrated the inhibition of multiple chemokines and cytokines in allografts from TNF-R1$^{-/-}$ recipients including RANTES, IFN-inducible protein-10, lymphotactin, and IL-1R antagonist, as well as altered levels of chemokine receptors. We correlated gene expression with the physiologic process of allograft rejection using self-organizing maps and identified distinct patterns of gene expression in allografts from TNF-R1$^{-/-}$ recipients. These findings indicate that in our experimental system TNF-α and LTα exert profound immunoregulatory effects through TNF-R1. *The Journal of Immunology,* **2002, 168: 483–489.**

Data from multiple studies suggest that TNF-α and lymphotoxin (LT)[3]α are important modulators of allograft rejection (1, 2). Increased levels of TNF-α and LTα have been detected in rejecting allografts in both clinical studies and animal models (3–9), and inhibition of these cytokines has been associated with prolonged allograft survival (10–12). However, the mechanism(s) by which TNF-α and LTα mediate allograft rejection remains incompletely understood.

TNF-α and LTα (also known as TNF-β) are two members of the TNF family of ligands that mediate a wide variety of immune functions including cytotoxicity, inflammation, and apoptosis (13). Both TNF-α and LTα were initially identified over a decade ago based on cytotoxicity of tumor cells. Subsequently, their role as mediators of diverse inflammatory processes including septic shock, cachexia, endothelial activation, thrombosis, and cancer growth has been established. In vitro, TNF-α and LTα have been shown to exert similar biological effects, although TNF-α is more potent in most experimental systems. Consistent with this observation, both TNF-α and LTα bind to one of the same two receptors, TNF-R1 (CD120a, also known as p60 (human) and p55 (murine)), or TNF-R2 (CD120b, also known as p80 (human) and p75 (murine)).

TNF-R1 and TNF-R2 are members of the TNF/nerve growth factor receptor family. Both TNF-R1 and TNF-R2 can activate NF-κB, c-*jun* kinase, and activating protein-1 promoting cellular activation and survival; however, only TNF-R1 contains a death domain which can trigger apoptosis. Although the precise role of the two receptors has not been established, at least some of the functions they mediate are distinct. Because TNF-α and LTα bind the same two receptors, studies that inhibit either TNF-α or LTα are difficult to interpret due to overlapping or compensatory effects of the other ligand. Further complicating such studies is the fact that LTβ, which binds to the LTβR, is a heterotrimeric complex composed of both LTα and LTβ subunits; thus, inhibition of LTα by gene deletion or protein blockade results in a lack of activity of both LTα and LTβ. Therefore, we have chosen to investigate the effects of TNF-α and LTα by focusing on a single receptor, TNF-R1.

Mice with targeted genetic deletions of TNF-R1 (TNF-R1$^{-/-}$) have normal lymphocyte populations and effective clonal deletion of self-reactive thymocytes (14, 15). However, TNF-R$^{-/-}$ mice demonstrate abnormalities in germinal center formation, follicular dendritic cell development, and IgG responses (16–18). In addition, they are resistant to endotoxic shock but susceptible to infection by *Listeria monocytogenes* (15). In studies of corneal transplants in TNF-R1$^{-/-}$ recipients, allograft survival was not prolonged when donor and recipient strains had complete MHC mismatches (19). However, there was a modest prolongation of allograft survival in strain combinations that had only minor Ag

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Received for publication December 22, 2000. Accepted for publication November 11, 2001.

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[1] This work was supported by grants from the American Heart Association, Arthritis Foundation, and the National Institutes of Health (AI44085) to D.L.P.

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[3] Abbreviations used in this paper: LT, lymphotoxin; RPA, RNase protection assay; m, murine; Ltn, lymphotactin; MIP, macrophage inflammatory protein; IP-10, IFN-inducible protein-10; MCP-1, monocyte chemoattractant protein-1; TCA-3, T cell activation-3; IL-1RA, IL-1R antagonist.

0022-1767/02/$02.00

mismatches. To investigate the role of TNF-R1 immunoregulation in the rejection of vascularized solid organ allografts, we performed heterotopic cardiac transplants in TNF-R1$^{-/-}$ recipients. Because the allografts were harvested from wild-type donors, expression of TNF-R1 by graft tissue was normal in both TNF-R1$^{-/-}$ and wild-type recipients. Thus, in our experimental system direct effects of TNF-R1 engagement by TNF-α and/or LTα, including direct cytotoxicity, on donor cells of the allograft were not inhibited. Similarly, TNF-α and/or LTα signaling through TNF-R2 in all tissues was unaffected. However, the immunoregulatory effects of TNF-R1 engagement, such as TNF-R1-modulated cytokine or chemokine production by nonallograft cells, were absent in TNF-R1$^{-/-}$ recipients.

The focus of this study was the effect on allograft rejection of immunomodulatory signals mediated by TNF-R1 expressed by recipient cells. We found that cardiac allograft survival was significantly prolonged in TNF-R1$^{-/-}$ recipients. To determine the molecular basis for the delayed kinetics of rejection in the TNF-R1$^{-/-}$ recipients, we analyzed a panel of cellular markers, cytokines, chemokines, and chemokine receptors using an iterative cluster algorithm that generated self-organizing maps. Incorporating gene analysis with the functional outcome of allograft rejection, self-organizing maps identified clusters of genes including cytokines, chemokines, and chemokine receptors with expression profiles that correlated with the kinetics of rejection. Our findings indicate that TNF-α and LTα exert profound immunoregulatory effects after transplantation, including enhanced infiltration of CD8 T cells and modulated levels of cytokine, chemokine, and chemokine receptors.

Materials and Methods

Vascularized heterotopic cardiac transplantation

Murine hearts were transplanted heterotopically as previously described (20). Hearts were harvested from freshly sacrificed donors and were immediately transplanted into 8- to 12-wk-old recipients anesthetized by i.p. injection with 50 mg/kg pentobarbital sodium. Donor aorta was anastamosed to recipient abdominal aorta, and donor pulmonary artery was anastamosed to recipient vena cava. All surgical procedures were completed within 60 min. Clinical allograft function was assessed by the presence of a palpable heartbeat in the allograft. Loss of a palpable heartbeat correlates with the development of end-stage rejection as confirmed by histology. Donor hearts that did not beat immediately after reperfusion or that stopped beating within 24 h after transplantation were excluded from analysis (>95% of all grafts functioned at 24 h). Allografts were harvested at days 1 and 5 and were divided into equal sections for mRNA isolation and for tissue sections for histology and immunohistochemistry.

Mice

TNF-R1$^{-/-}$ mice were back-crossed >10 generations into the C57BL/6 background (The Jackson Laboratory, Bar Harbor, ME). Eight- to 12-wk-old male mice, including TNF-R1$^{-/-}$, BALB/cByJ (BALB/c) (H-2^d), and C57BL/6J (H-2^b) (all from The Jackson Laboratory) were used as donors and recipients in the transplant experiments. Mice were maintained in vented racks with constant temperature and humidity in our animal facility under antivirus Ab-free conditions.

Mixed lymphocyte reaction

MLR was performed as previously described (21). Responder spleen cells (2×10^5) were stimulated with 4×10^5 stimulator spleen cells, which had been irradiated with 2000 rad, in 200 μl of RPMI supplemented with 10% FCS. Twelve hours before harvesting, 1 μCi of [^{3}H]thymidine was added to the culture system. Cells were harvested at 96 h and thymidine incorporation was determined as described. Cultures were performed in quadruplicate and SEM were <10%.

Histology and immunohistochemistry

Transplanted hearts were harvested at days 1 and 5 after transplantation and fixed in 10% neutral buffered formalin for histologic examination. After dehydration and paraffin embedding, multiple 5- to 6-μm-thick sections were stained with H&E. Multiple sections of each heart were examined and the extent of rejection was quantified on a scale of 0–4 using a modified International Society of Heart and Lung Transplantation grading scale (22). Each heart was also graded on a scale of 0–4 according to the degree of ischemic healing. Ischemic healing scores were assigned as follows: grade 0, no healing ischemic injury present; grade 1, involvement of up to 25% of the myocardium by healing ischemic injury; grade 2, involvement of 25–50% of the myocardium; grade 3, involvement of 50–75% of the myocardium; grade 4, involvement of >75% of the myocardium.

For immunohistochemistry, 5-μm-thick cardiac tissue sections were cut from whole graft hearts that were snap-frozen in liquid nitrogen. The sections were fixed in acetone for 10 min, and nonspecific Ig binding was blocked with normal mouse serum diluted 1/10 in PBS with 2% BSA. A modified avidin-biotin immunoperoxidase method was used for immunostaining (23). The primary antisera were rat anti-CD8 and rat anti-CD4, both diluted 1/25 in PBS/BSA (both antisera from BD PharMingen, San Diego, CA). Slides were incubated in their respective primary antisera overnight at 4°C, followed by the secondary Ab, biotinylated mouse-anti-rat IgG, diluted 1/500 in 5% milk in PBS (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at 4°C. Endogenous peroxidases were blocked by 10% hydrogen peroxide in methanol for 10 min at room temperature followed by a 30-min incubation in streptavidin-HRP, diluted 1/1000 in PBS (Jackson ImmunoResearch Laboratories). Immunopositivity was visualized with 0.025% diaminobenzidine and the slides were counterstained with 2% methyl green (both from Sigma-Aldrich, St. Louis, MO). Images were obtained using a Leica DMLB microscope, interfaced with image analysis software (Leica Q500IW; Leica, Bannockburn, IL).

RNase protection assay

Total mRNA was isolated from transplanted hearts with TriReagent according to the protocol supplied by the manufacturer (Sigma-Aldrich). mRNA was also isolated from untransplanted hearts from unmanipulated BALB/c mice as additional control samples. Gene expression was analyzed using the RiboQuant MultiProbe mRNase protection assay (RPA) system according to the manufacturer's instructions (BD PharMingen). A total of 15 μg of mRNA was used per hybridization and mRNase reaction. mRNA was hybridized with ^{35}S-labeled probes. After mRNase treatment and purification, protected probes were electrophoresed on a denaturing 5% polyacrylamide gel. Gels were exposed in a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The identity of each protected fragment was established by analyzing its migration distance against a standard curve of the migration distance vs the log nucleotide length for each undigested probe. Samples were normalized to the housekeeping gene, GAPDH. Protected bands were quantitated by densitometry using ImageQuant software (Molecular Dynamics). The following templates were used: murine (m)CK-5 (lymphotactin (Ltn), RANTES, eotaxin, macrophage inflammatory protein (MIP)-1β, MIP-1α, MIP-2, IFN-inducible protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), T cell activation-3 (TCA-3), L32, GAPDH), mCK-2b (IL-12p35, IL-12p40, IL-10, IL-1α, IL-1β, IL-1R antagonist (IL-1RA), IL-18, IL-6, IFN-γ, macrophage inflammatory factor (MIF), L32, GAPDH), mCK-3b (TNF-β/LTα, LTβ, TNF-α, IL-6, IFN-γ, IFNβ, TGFβ1, TGFβ2, TGFβ3, MIF, L32, GAPDH), mCR-5 (CCR1, CCR1b, CCR4, CCR5, CCR2, L32, GAPDH), mCR-6 (inducible NO synthase, CXCR2, CXCR4, BLR-1, CCR8a, CCR8b, CXCR3, CCR6, L32, GAPDH), and mCD-1 (TCRγ, TCRα, CD3ϵ, CD4, CD8α, CD8β, CD19, F4/80, CD45, L32, GAPDH).

Serum cytokine ELISA

Blood was obtained by cardiac puncture from transplanted mice at the time of sacrifice. Serum was aliquoted and stored at −80°C. Samples were thawed and serum concentrations of TNF-α, IL-1β, IFN-γ, and IL-6 protein were determined using standard ELISA kits according to the manufacturer's instructions (pooled samples, n = 2–3 per group; all from Quantikine, Minneapolis, MN). All protein measurements were performed in triplicate.

Self-organizing maps

Self-organizing maps were generated by GeneCluster (Whitehead Institute for Biomedical Research, Cambridge, MA) with normalization using a 2 × 2 geometry of four seed maps (24). Four maps were selected empirically to eliminate clusters with few genes or large SDs. The centroids and SDs of the groupings were analyzed using 10,000 epochs. Additional epochs did not alter the gene clusters of the maps.

Statistical analysis

Allograft survival data were calculated as mean days ± SD. For survival and serum cytokine data, comparisons between groups were performed

using the paired t test, and differences were considered significant at $p < 0.05$.

Results

Cardiac allografts transplanted into TNF-R1$^{-/-}$ recipients have prolonged survival

To investigate the hypothesis that signals transduced via TNF-R1 are crucial for an allogeneic response, we first analyzed MLR. Consistent with previous reports, TNF-R1$^{-/-}$ responder cells had significantly reduced proliferative responses compared with wild-type cells when stimulated with allogeneic splenocytes ($p = 0.004$; Fig. 1) (25). To investigate this response in vivo, we performed heterotopic cardiac transplants using wild-type BALB/c donors and TNF-R1$^{-/-}$ (C57BL/6 background) and wild-type C57BL/6 recipients (Table I). Allograft rejection, determined by the loss of a palpable heartbeat and confirmed by histology, was significantly delayed in the TNF-R1$^{-/-}$ recipients compared with wild-type recipients ($p < 0.00001$). Because expression of TNF-R1 was normal in the transplanted hearts, TNF-α and LTα could still exert direct effects on the allograft through interaction with TNF-R1. Thus, the prolonged allograft survival observed in TNF-R1$^{-/-}$ recipients was not due to a lack of direct TNF-R1-mediated effects including cytotoxicity on the grafts, nor was this effect due to a lack of TNF-α/LTα signaling altogether, because TNF-R2 was normally expressed in both experimental groups. Rather, our findings indicate that the enhanced allograft survival in TNF-R1$^{-/-}$ recipients was due to a lack of TNF-α/LTα immunomodulatory effects, mediated by TNF-R1 on recipient cells.

Allografts from TNF-R1$^{-/-}$ recipients have increased cellular infiltration

To determine the effects of TNF-R1 deficiency on cellular infiltration and graft rejection, we examined the histology of allografts from TNF-R1$^{-/-}$ and wild-type recipients at days 1 and 5 after transplantation. We analyzed early time points following transplantation to avoid confounding effects of clinical rejection and allograft failure. At day 1 there were no significant differences in histology between the TNF-R1$^{-/-}$ and wild-type groups (data not shown). Rejection and ischemia scores were 0 for grafts from both experimental groups at this time point. By day 5, infiltration of inflammatory cells could be detected in allografts from both TNF-R1$^{-/-}$ and wild-type recipients (Fig. 2, *B* and *C*). Ischemia scores remained 0 in grafts from both groups. Interestingly, allografts from TNF-R1$^{-/-}$ recipients had higher rejection scores (3 vs 2) and increased mononuclear cell infiltration compared with wild-type recipients.

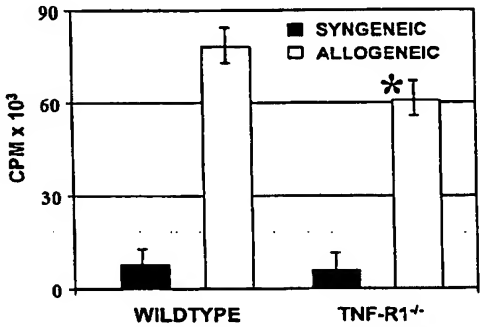

FIGURE 1. Splenocytes from TNF-R1$^{-/-}$ mice have reduced proliferative responses to allogeneic cells. MLR was performed as described in *Materials and Methods*. Splenic stimulator cells from wild-type C57BL/6 (B6) (Syngeneic) and BALB/c (Allogeneic) mice were irradiated with 2000 rad and incubated with responder splenocytes from wild-type B6 and TNF-R1$^{-/-}$ mice for 96 h. [^{3}H]Thymidine was added 12 h before harvesting and proliferation was determined by thymidine incorporation expressed as counts per minute $\times 10^3$ as shown on the y-axis ($p = 0.0004$). All cultures were performed in quadruplicate.

Allografts from TNF-R1$^{-/-}$ recipients have increased CD8 cellular markers

To investigate the cellular composition of intragraft infiltrates, we analyzed molecular markers of T cells, B cells, and macrophages in allografts with RPA. Levels of mRNA for all cellular markers were low at day 1, but increased by day 5 following transplantation in both the TNF-R1$^{-/-}$ and wild-type recipients (Fig. 3). As expected, levels were also low (<2% of GAPDH) for all cellular markers analyzed in untransplanted BALB/c hearts (data not shown). At day 5, allografts from TNF-R1$^{-/-}$ recipients had markedly increased levels of mRNA for the T cell markers TCRα and CD3ϵ, and for the specific CD8 T cell markers CD8α and CD8β (Fig. 3, *A* and *C*). Levels of CD4, TCRδ, and CD19 mRNA were low in both groups, suggesting that in this experimental system CD4$^+$ T cells, $\gamma\delta$ T cells, and B cells do not contribute significantly to infiltrates at early time points after transplantation.

These findings indicate that immunomodulatory signals mediated by TNF-R1 influence the specific populations of infiltrating cells present in the allograft by day 5. Specifically, these results suggest that in the absence of TNF-R1-mediated immunomodulatory signals, early graft infiltrates are composed of a greater percentage of CD8 T cells. To confirm protein expression of the T cell markers, we performed immunohistochemistry for CD8 and CD4 cellular markers in allografts from TNF-R1$^{-/-}$ and wild-type recipients at day 5 posttransplantation (Fig. 2, *D–I*). Consistent with the mRNA transcript levels, the infiltrating cells demonstrated a greater abundance of immunostaining for CD8 than for CD4.

Levels of acute phase response cytokines are not different in allografts from TNF-R1$^{-/-}$ recipients

To determine whether cellular infiltration was modulated by early cytokine production, we first analyzed levels of mRNA for the acute phase response cytokines IL-1β, TNF-α, IL-6, and LTα, plus IL-1RA and IFN-γ (26). Increased levels of these cytokines, which may be modulated by signals mediated by TNF-R1, have been associated with rejection in previous studies (1, 2, 27–29). However, we found similar levels of IFN-γ, IL-1β, TNF-α, IL-6, and LTα mRNA in both experimental groups at day 1 posttransplantation (Fig. 4*A*). IL-1RA mRNA levels were greater in allografts from wild-type recipients at this initial time point, but by day 5 levels of mRNA for IL-1RA and the acute phase response cytokines were similar in both groups, while IFN-γ levels were elevated in the TNF-R1$^{-/-}$ group (Fig. 4*B*). In untransplanted BALB/c hearts mRNA levels of all of these cytokines were <2% of GAPDH (data not shown).

To determine whether intragraft mRNA levels correlated with circulating protein levels, we analyzed serum protein concentrations of IFN-γ, IL-1β, TNF-α, and IL-6 in TNF-R1$^{-/-}$ and wild-type recipients (Fig. 4, *C* and *D*). At day 1 the serum concentrations of TNF-α and IL-6 were significantly elevated in TNF-R1$^{-/-}$ recipients, while IL-1β levels were higher in wild-type recipients. Circulating IFN-γ levels were low in both experimental groups at this time point. At day 5, both IFN-γ and IL-1β levels were significantly higher in the wild-type group, while there was no longer a significant difference in TNF-α or IL-6 levels.

These results suggest that TNF-R1-mediated immunomodulatory signals are not critical early negative regulators of TNF-α, LTα, IL-1β, IL-6, or IL-RA mRNA expression in the allograft but

Table 1. *TNF-R1$^{-/-}$ recipients have prolonged cardiac allograft survival*

Experimental Group	Donor Strain	Recipient Strain	Allograft Survival (days)	MST ± SD
Wild type	BALB/c (H-2^d)	C57BL/6 (H-2^b)	7, 7, 8, 8, 9, 10	8.17 ± 5.71
TNF-R1$^{-/-}$	BALB/c (H-2^d)	C57BL/6 (H-2^b) TNF-R1$^{-/-}$	31, 32, 38, 40, 43, 45	38.16 ± 1.70

[a] Heterotopic cardiac transplants were performed as described in *Materials and Methods*. Allograft survival was assessed as the number of days that a pulse could be palpated in the allograft. MST, Mean survival time in days of allografts in each experimental group ($n = 6$ in each group; $p < 0.00001$).

may delay the up-regulation of intragraft IFN-γ expression. Interestingly, the prolonged allograft survival demonstrated in TNF-R1$^{-/-}$ recipients also indicates that TNF-α, LTα, IL-1β, IFN-γ, and IL-6 are not sufficient to mediate early allograft rejection in this experimental system. The finding that serum protein concentrations of IFN-γ, IL-1β, TNF-α, and IL-6 were altered in TNF-R1$^{-/-}$ recipients, despite similar intragraft mRNA levels of most of these cytokines in both groups, suggests that nonallograft cells from organs such as spleen, lymph node, and liver are the principal sources of circulating levels of these cytokines.

Levels of chemokines are altered in allografts from TNF-R1$^{-/-}$ recipients

Studies from multiple laboratories have suggested that chemokines are important mediators of allograft rejection, and TNF-α is a well-described stimulus for chemokine production by many cell types (30, 31). Therefore, we analyzed levels of mRNA for Ltn, RANTES, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1, and TCA-3 in allografts. Levels of mRNA for all chemokines studied were low in both experimental groups at day 1 with the exception of MIP-2, which was elevated in the wild-type group (Fig. 5*A*). However, by day 5 there were higher levels of RANTES, IP-10, and Ltn mRNA and lower levels of MIP-1β, MIP-1α, MCP-1, and TCA-3 in grafts from wild-type recipients (Fig. 5*B*). Levels of mRNA for all chemokines studied were <4% of GAPDH in untransplanted BALB/c hearts (data not shown).

Levels of chemokine receptors are altered in allografts from TNF-R1$^{-/-}$ recipients

To investigate whether levels of chemokine mRNA in the TNF-R1$^{-/-}$ recipients were associated with altered chemokine receptor expression, we analyzed levels of a panel of chemokine receptors. At day 1, levels of mRNA for all chemokine receptors studied were lower in the TNF-R1$^{-/-}$ group than in the wild-type group

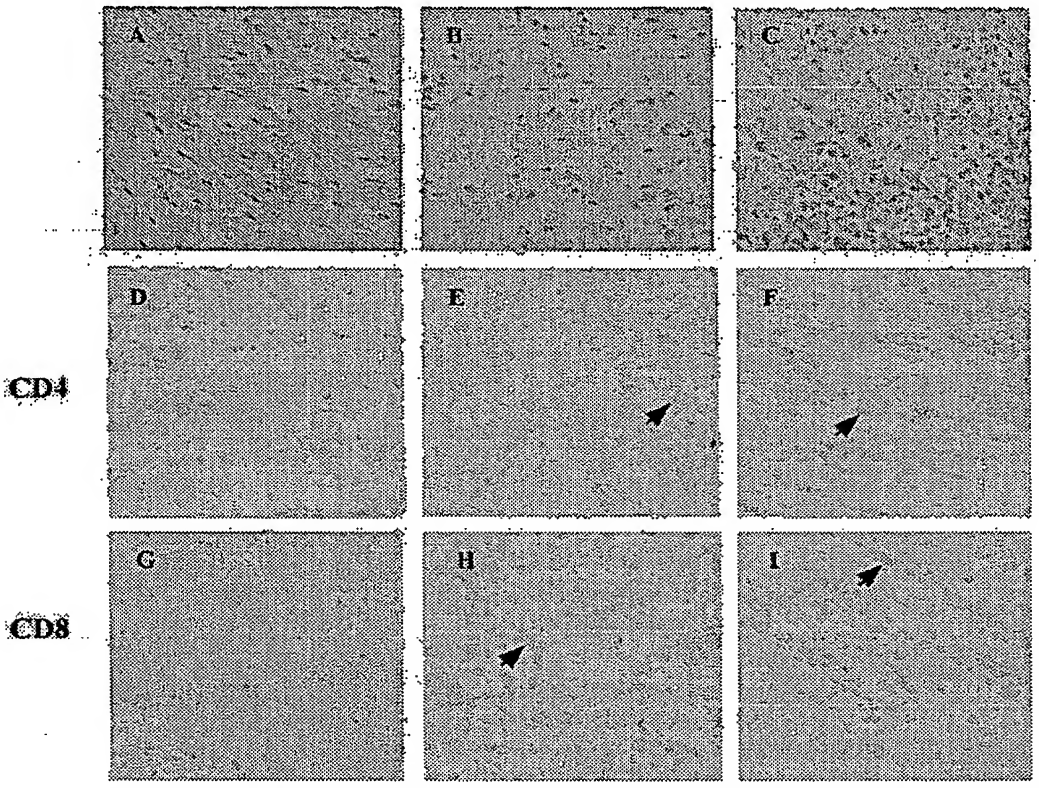

FIGURE 2. Allografts from TNF-R1$^{-/-}$ recipients have increased cellular infiltrates. Allografts were harvested and prepared for histologic and immunohistochemical analysis as described in *Materials and Methods*. *A–C*, Untransplanted (BALB/c) heart demonstrates normal histology without leukocytic infiltrates (*A*). At day 5 the allograft from the wild-type recipient demonstrates grade 2 rejection (*B*), while the allograft from the TNF-R1$^{-/-}$ recipient demonstrates grade 3 rejection (*C*). Ischemic recovery scores were 0 in allografts from both experimental groups at days 1 and 5. All figures were originally photographed at ×400 magnification. *D–I*, Immunohistochemistry for CD4 and CD8 cellular markers was performed as described in *Materials and Methods*. Untransplanted hearts (*D* and *G*), graft hearts from wild-type recipients at day 5 (*E* and *H*), and graft hearts from TNF-R1$^{-/-}$ recipients at day 5 (*F* and *I*) were stained by indirect immunoperoxidase as described. Arrows indicate representative cells with positive staining. All figures were originally photographed at ×20 magnification.

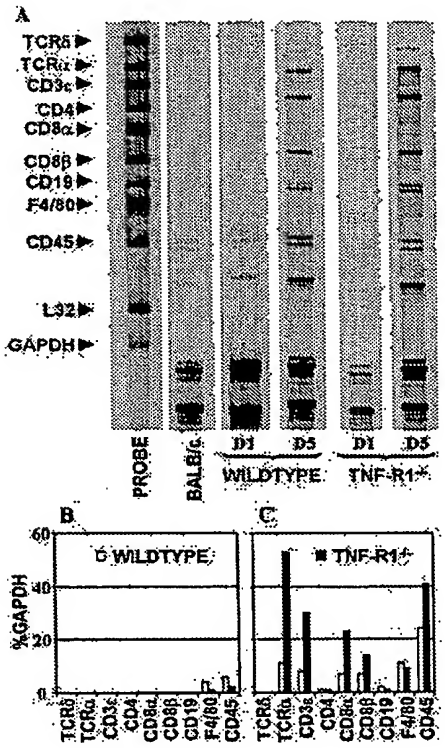

FIGURE 3. CD8 T cell surface marker mRNA levels are modulated in allografts from TNF-R1$^{-/-}$ recipients. mRNA was isolated from allografts and analyzed by RPA as described in *Materials and Methods*. Representative sections of an RPA gel are shown in *A*. Bands in the sample lanes were identified by analyzing their migration distance against a standard curve of migration distance vs the log nucleotide length of each undigested probe. Bands from untransplanted (BALB/c) heart, allografts from wild-type recipients at days 1 (D1) and 5 (D5), and allografts from TNF-R1$^{-/-}$ recipients at days 1 (D1) and 5 (D5) were quantitated by densitometry. Cellular marker mRNA levels in allografts from wild-type and TNF-R1$^{-/-}$ recipients were expressed as a percentage of the housekeeping gene GAPDH and depicted graphically at days 1 (*B*) and 5 (*C*) posttransplantation.

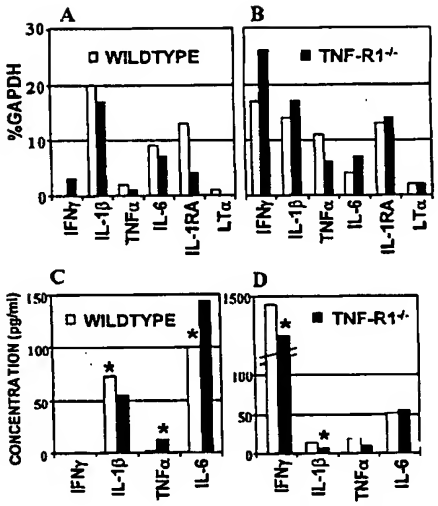

FIGURE 4. Levels of acute phase response cytokines are not altered in allografts from TNF-R1$^{-/-}$ recipients. *A* and *B*, mRNA isolation and RPA analysis were performed as described in *Materials and Methods*. Acute phase response cytokines, IFN-γ, and IL-1RA mRNA levels in allografts from wild-type and TNF-R1$^{-/-}$ recipients were measured at days 1 (*A*) and 5 (*B*). mRNA levels were quantified by densitometry and expressed as a percentage of GAPDH. *C* and *D*, Serum cytokine protein levels were determined by ELISA as described in *Materials and Methods* at days 1 (*C*) and 5 (*D*). IL-1β, day 1, $p = 0.015$; IL-6, day 1, $p = 0.002$; TNF-α, day 1, $p = 0.0029$; IFN-γ, day 5, $p = 0.0002$; IL-1β, day 5, $p = 0.0037$.

(Fig. 6*A*). On day 5, levels of mRNA for CCR1 and CCR2 were higher in grafts from TNF-R1$^{-/-}$ recipients (Fig. 6*B*). CXCR4 levels remained higher in allografts from wild-type recipients, CCR5 levels were similar in both groups, and CCR1b, CCR3, and CCR4 mRNA levels were detectable only in the wild-type group and only at low levels at day 5. Levels of mRNA for all chemokine receptors studied were <3% of GAPDH for untransplanted BALB/c hearts (data not shown). These results suggest that in the absence of TNF-R1-mediated immunomodulatory signals, expression of CCR1 and CCR2 is increased by day 5.

Genes are modulated in distinct patterns in allografts from TNF-R1$^{-/-}$ recipients

To identify the molecular basis by which TNF-R1-mediated signaling promotes allograft rejection, we used self-organizing maps to cluster distinct patterns of gene expression in allografts from TNF-R1$^{-/-}$ and wild-type recipients. Self-organizing maps are artificial neural network algorithms that organize data into clusters with similar expression profiles (24). We generated four maps using a 2 × 2 geometry with 10,000 epochs to analyze expression profiles (Fig. 7). Maps 0 and 2 contain genes that are increased in both TNF-R1$^{-/-}$ and wild-type groups at day 5. However, the magnitude of increase is greater in the wild-type group in map 2, but greater in the TNF-R1$^{-/-}$ group in map 0. These quantitative differences may contribute to the distinct kinetics of rejection in the two groups. Importantly, maps 1 and 3 identify gene clusters with patterns of expression that are qualitatively different at day 1 following transplantation. Both map 1 and map 3 contain genes that are not up-regulated in the TNF-R1$^{-/-}$ group at day 1 following transplantation. These clusters identify genes that are not rapidly induced in the absence of TNF-R1 and may be critical for optimal allograft rejection.

Discussion

Our study examined the immunoregulatory effects of TNF-R1 signals in recipient cells on the development of allograft rejection. Our data support the hypothesis that signals by TNF-α and LTα mediated by TNF-R1 regulate the mononuclear cell infiltration in the allograft as well as the expression of a subset of cytokines, chemokines, and chemokine receptors. Interestingly, we found that

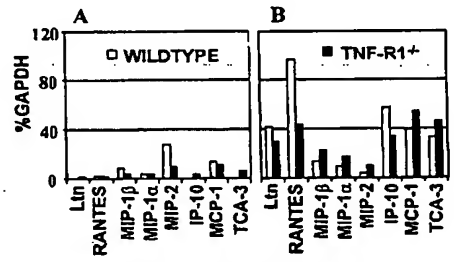

FIGURE 5. Chemokine mRNA levels are altered in allografts from TNF-R1$^{-/-}$ recipients. mRNA was isolated from allografts and analyzed by RPA as described in *Materials and Methods*. Chemokine mRNA levels from wild-type and TNF-R1$^{-/-}$ recipients at days 1 (*A*) and 5 (*B*) posttransplantation were measured by densitometry and expressed as a percentage of GAPDH.

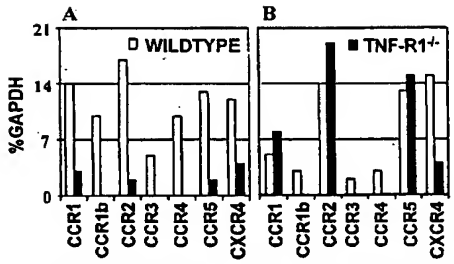

FIGURE 6. Chemokine receptor mRNA levels are altered in allografts from TNF-R1$^{-/-}$ recipients. Isolation of mRNA and analysis by RPA were performed as described in *Materials and Methods*. Chemokine receptor mRNA levels in allografts from wild-type and TNF-R1$^{-/-}$ recipients at days 1 (*A*) and 5 (*B*) were quantitated by densitometry and shown as a percentage of GAPDH.

allograft survival was prolonged in grafts from TNF-R1$^{-/-}$ recipients despite increased infiltration of mononuclear cells and increased markers of CD8 T cells in the early posttransplant period.

These findings suggest that in the absence of TNF-R1-mediated immunomodulatory signals, inflammatory cells are efficiently recruited to the allograft but are less effective mediators of rejection. It is possible that unopposed signaling through TNF-R1, which was intact in both experimental groups, may have contributed to the prolonged allograft survival in the TNF-R1$^{-/-}$ group, an explanation suggested by the finding by Yamada and colleagues (19) that survival of corneal transplants in mice lacking TNF-R2 was reduced compared with corneal transplants in either wild-type recipients or recipients lacking both TNF-R1 and TNF-R2. Based on immunohistochemistry and RPA, we determined that infiltrates in the allografts from TNF-R1$^{-/-}$ recipients were composed predominantly of CD8 T cells. Increased CD8 T cell infiltration in the TNF-R1$^{-/-}$ group may be influenced by differences in local chemokine activity, expression of chemokine receptors, or altered endothelial activation state, all factors know to be modulated by TNF-α and LTα. Interestingly, we detected low levels of CD4$^+$ lymphocytes, $\gamma\delta$ T cells, and B lymphocytes in allografts from both

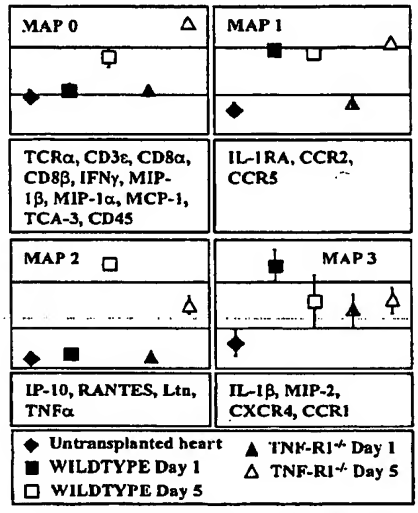

FIGURE 7. Allografts from TNF-R1$^{-/-}$ recipients have distinct gene expression patterns. Four self-organizing maps (maps 0–4) were generated using GeneCluster software as described in *Materials and Methods*. Genes contained in each map are identified in the corresponding text box. All genes were mapped using an arbitrary reference scale on the *y*-axis.

groups, suggesting that in this experimental model CD8$^+$ lymphocytes and macrophages were the principal effector cells at day 5.

We also demonstrated reduced levels of the chemokines RANTES, IP-10, and Ltn and elevated levels of MIP-1β, MIP-1α, MCP-1, and TCA-3 in allografts from TNF-R1$^{-/-}$ recipients. These results suggest that the expression of these chemokines is regulated, either directly or indirectly, by TNF-R1-mediated signals. Elevated levels of RANTES, IP-10, and Ltn have been demonstrated in allograft rejection in human and experimental models, and regulation of the production of these mediators through TNF-R1 immunomodulatory signals may be an important mechanism by which TNF-α and LTα regulate effector functions in allograft rejection (32–37). Interestingly, there is evidence that RANTES, IP-10, and Ltn may be preferentially expressed by activated CD8 T cells, and there are also data suggesting that IP-10 may be important for CD8 T cell-mediated antitumor and antiviral effector functions (38–42). Our finding of reduced levels of these chemokines in allografts from TNF-R1$^{-/-}$ recipients despite robust levels of CD8 T cells suggests that CD8 T cells in TNF-R1$^{-/-}$ mice may have an impaired ability to produce these chemokines.

We found that levels of mRNA for CCR1, CCR1b, CCR2, CCR3, CCR4, and CCR5 were not up-regulated in allografts from TNF-R1$^{-/-}$ recipients 1 day after transplantation, suggesting that TNF-R1-mediated immunomodulatory signals are important for the optimal expression of these receptors immediately after transplantation. The increase in levels of CCR1 and CCR2 observed in allografts from TNF-R1$^{-/-}$ recipients at day 5 suggests that the absence of TNF-R1 immunomodulatory signals delays but does not prevent up-regulation of these chemokine receptors. Enhanced expression of many chemokine receptors has been associated with allograft rejection (31, 43), and early signaling by chemokines through these receptors may contribute to the more rapid rejection observed in allografts from wild-type recipients.

Surprisingly, we did not find substantial differences in intragraft mRNA levels for the acute phase cytokines TNF-α, LTα, IL-1β, and IL-6. We did note reduced IL-1RA and increased IFN-γ levels in allografts from TNF-R1$^{-/-}$ recipients, indicating that TNF-R1-mediated signaling modulates the expression of these mediators within the allograft. We also detected significant differences in serum protein levels of IFN-γ, IL-1β, TNF-α, and IL-6 in TNF-R1$^{-/-}$ and wild-type recipients, suggesting that TNF-R1-mediated immunomodulatory signals influence the expression of these cytokines by nonallograft cells. The observation that intragraft TNF-α and LTα mRNA levels were similar in wild-type and TNF-R1$^{-/-}$ recipients raises the possibility that negative feedback signals for these cytokines may be transmitted through TNF-R2, or alternatively that local signaling through TNF-R1 on donor allograft cells may be sufficient to reduce intragraft levels of these cytokines.

We analyzed the functional outcome of allograft rejection in the context of global gene expression analysis, using self-organizing maps to identify subsets of genes that were differentially regulated in the TNF-R1$^{-/-}$ and wild-type recipients. Importantly, at day 1 following transplantation, the genes in maps 1 and 3 were up-regulated in the wild-type, but not the TNF-R1$^{-/-}$, recipients, suggesting that they are crucial in the early rejection response. The genes in map 2 were up-regulated in both groups at day 5 but were higher in the wild-type group, suggesting that elevated levels of these genes may be important for optimal allograft rejection to occur. Conversely, at day 5, the genes in map 0 were expressed at greater levels in the TNF-R1$^{-/-}$ group, suggesting that they are not sufficient for the acute rejection response and may contribute to the delayed kinetics of rejection observed in the TNF-R1$^{-/-}$ group.

Our findings support the conclusion that TNF-R1-mediated immunomodulatory signals regulate key early steps in the development of allograft rejection. Through our analysis of gene expression, correlated with the physiological outcomes following transplantation, we identified distinct subsets of genes that are dependent on TNF-R1-mediated immunomodulatory signals immediately after transplantation. Importantly, our results indicate that these genes may be critical for optimal allograft rejection. Determining how TNF-α/LTα signaling through TNF-R1 contributes to allograft rejection may facilitate the development of therapeutic strategies to prolong allograft survival.

Acknowledgments

We thank Min Xu, Michael Buckley, and Walter Zybko for their technical support and Melissa Kurkjian for her help with manuscript preparation.

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